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Characterization of *Vibrio parahaemolyticus* isolated from farmed sea bass (*Dicentrarchus labrax*) during disease outbreaks

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Abstract

Vibrio parahaemolyticus was isolated from the internal organs of diseased sea bass (*Dicentrarchus labrax*) cultured in a marine cage farm and an inshore fish farm in the summer 2010. All strains isolated from diseased sea bass were tested for virulence by intraperitoneal injection. The isolates ($n = 6$) were pathogenic for sea bass. Only bacterial strains showing haemolytic activity were virulent. LD50 values ranged from 3.52×10^4 to 2.29×10^6 cfu fish^{-1}. In this study, we report the characterization and the virulence of *V. parahaemolyticus* strains isolated from sea bass originating from two different fish farms (marine cage farm and inshore fish farm in Tunisia).

Keywords: *Vibrio parahaemolyticus*; Outbreaks; Virulence properties

Background

Vibrio parahaemolyticus is part of the natural estuarian microflora and coastal marine waters and can be present in seafood, especially shellfish and bivalve mollusks (DePaola et al. 2003; Zorrilla et al. 2003). Starting from 2000, an increase of outbreaks associated with the 03:K6 serovar has been observed in Asia (Matsumoto et al. 2000), North America and Chile (Martinez-Urtaza et al. 2005). This serovar was also reported from outbreaks or sporadic cases in Europe, France and Spain (Martinez-Urtaza et al. 2005; Quilici et al. 2005), and Africa and Russia (Nair et al. 2007).

The *Vibrio* pathogenic species produce various virulence factors including enterotoxin, haemolysin, cytotoxin, protease, lipase, phospholipase, siderophore, adhesive factor and/or haemagglutinins (Zhang and Austin 2005). The virulence of *V. parahaemolyticus* strains is commonly associated with expression of thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH), which are encoded by the *tdh* and *trh* genes. Therefore, the *tdh* gene, marked by a β-type haemolysis on Wagatsuma agar (Nishibuchi and Kaper 1995), and the *trh* gene, correlated to a positive urease tests (Okuda et al. 1997), serve as markers for pathogenic strains. Many extracellular proteases are suggested to play important roles in virulence of *Vibrio spp*. In a recent study (Lee et al. 2002), a protease was identified as the major virulence factor in a clinical *tdh* and *trh* negative *V. parahaemolyticus* isolate, and this enzyme showed cytotoxic activity on CHO and Vero cells. In this study, we reported the characterization of six *V. parahaemolyticus* isolated during outbreaks affecting sea bass (*Dicentrarchus labrax*) isolated in two different fish farms

(inshore and offshore fish farms), and we demonstrate its implication in the disease experimental challenges.

Methods

Sampling and bacterial isolation

Juvenile (weight 5 to 7 g, length 8 to 10 cm) and adults (weight 100 to 120 g, length 14 to 16 cm) *D. labrax* were obtained from two different fish farm, marine cage farm and inshore fish farm in summer 2010. Ten fish were collected from each size and each site (20 juveniles and 20 adults). The two fish farms are located in the biggest centre in Tunisia (Sousse, Centre of Tunisia). Samples from liver, spleen, kidney and external lesions of several fish from marine cage farm and inshore fish farm were cultured on alkaline peptone water (1% NaCl, pH 8.6) and incubated at 37°C for 18 to 24 h. A loopful of the enrichment culture was streaked onto thiosulphate-citrate-bile salt-sucrose agar used for the selective isolation of *Vibrio* strains (TCBS agar, Scharlau Microbiology, Spain). After 18 to 24 h of incubation at 37°C, the cultures giving pure green colonies were randomly selected then subcultured on tryptic soy agar (TSA, Difco, Spain) supplemented with 1% NaCl according to the protocol described by Hara-Kudo et al. (2001). The isolated bacteria were frozen at −80°C with 20% (*v*/*v*) glycerol for further analysis.

Phenotypic characterization of bacterial strains

Standard procedures described in Bergey's Manual of Systematic Bacteriology (Alsina and Blanch 1994) were used for the species identification. Gram coloration, cell morphology, the oxidase, catalase, indole production, O-F test, motility (mannitol-motility agar, Pronadisa, Madrid, Spain) and susceptibility to the vibriostatic compound O/129 (10 and 150 μg/disc) were the first tests employed to identify the organisms belonging to the *Vibrio* genus. The API 20NE (bioMerieux, Marcy l'Etoile, France) procedure was modified in order to incorporate a 2.5% NaCl concentration in all microtubes. An initial bacterial suspension was therefore prepared in 5 mL of a 2.5% NaCl solution instead of the recommended 0.85% NaCl medium. Incubation time and temperature were maintained within the limits prescribed (30°C for 24 h) (Croci et al. 2007). Identification was obtained through the APILAB PLUS software (bioMerieux) and was considered acceptable when giving a probability equal or greater than 80% (Croci et al. 2007).

Halophilism tests were performed using tryptone broth composed of casein enzymic hydrolysate and different concentrations of NaCl (0%, 3%, 6%, 8% and 10% *w*/*v*). Additional characterization tests for the identification of *V. parahaemolyticus* (Hormansdorfer et al. 2000), arginine dehydrolase tests and O/129 susceptibility tests were performed (FDA and Drug Administration 1992). The reference strains listed in Table 1 were used as positive controls.

Confirmation of the species level by detection of *toxR* gene

DNA minipreparations were made from each strain using the Wizard genomic DNA purification kit (Promega, Madison, WI, USA). The DNA was later resuspended in TE (10 mM Tris-HCl, 100 mM EDTA, pH 7.8) buffer and stored at −20°C. The quantity of genomic DNA in each sample was measured at 260 nm using a spectrophotometer (Ultraspec 2100 Pro; Amersham Biosciences Europe GmbH, France). The DNA

Table 1 Biochemical and morphological characterization of isolates from sea bass *D.* and *V. parahaemolyticus* (ATCC 17802 and ATCC43996)

Tests	Strains ($n = 6$)	*V. parahaemolyticus* ATCC17802	*V. parahaemolyticus* ATCC43996
Growth on TCBS	Green	Green	Green
Gram stain	–	–	–
Cytochrome oxidase	+	+	+
0/129 sensitivity	+	+	+
Swarming	–	–	–
Growth at% NaCl	+	+	+
0%	–	–	–
3%	+	+	+
6%	+	+	+
8%	+	+	+
10%	+	+	+
Growth at	+	+	+
4°C	–	–	–
25°C	+	+	+
37°C	+	+	+
40°C	+	+	+
β-galactosidase (ONPG test)	+	–	–
Arginine dihydrolase	+	–	–
Lysine decarboxylase	+	+	+
Ornithine decarboxylase	+	+	+
Citrate	–	–	–
Production of H_2S	–	–	–
Urease	–	+	–
Tryptophan deaminase	V	+	+
Indol	V	+	+
VP test	–	–	–
Gelatin liquefaction	+	+	+
Acid from	+	+	+
Glucose	+	+	+
Mannitol	+	+	+
Inositol	–	–	–
Sorbitol	–	–	–
Rhamnose	–	–	–
Sucrose	–	–	–
MeLibiose	–	–	–
Amygdalin	–	–	–
Arabinose	+	+	+
Lactose	–	–	–
Cellobiose	–	–	–

V, variable; ONPG, ortho-nitrophenyl-β-galactoside; VP, Voges-Proskauer test; +, positive; –, negative.

concentration of each sample was adjusted to (40 ng/μL) for polymerase chain reaction (PCR) by diluting DNA in distilled deionized water.

V. parahaemolyticus identification was confirmed with the presence of the outer membrane protein regulation operon gene (*toxR*) (Kim et al. 1999). The reaction for *toxR* analysis was performed in a total volume of 25 μL containing 2.5 μL of DNA sample, 15.25 μL of deionized water, 2.5 μL of 10 × buffer, 2 μL of 25 mM concentration of $MgCl_2$, 0.5 μL of 10 mM concentration of dNTP, 1 μL (10 μM) of each primer (ToxR-F GTCTTCTGACGCAATCGTTG and ToxR-R ATACGAGTGGTTGCTGTCATG) and 0.25 μL of 5 U/μL of Taq DNA polymerase (Promega). The PCR assay was performed at 94°C for 5 min followed by cycles of 94°C for 1 min, 63°C for 1 min and 72°C for 2 min in a DNA thermal cycler (Kim et al. 1999). The PCR fragments were subjected to electrophoresis on a 1.5% agarose gel in Trisborate-EDTA buffer (0.89 mol/L Tris, 0.89 mol/L boric acid, 0.02 mol/L EDTA, pH 8.0), visualized by ethidium bromide staining and photographed using Gel Doc XR apparatus (Biorad, Hercules, CA, USA).

Serum sensitivity and exoenzyme productions

Enzymatic activities of isolates were analysed by spot inoculations on TSA 2% NaCl supplemented with skimmed milk (2% *w*/*v*), Tween 80 (1% *w*/*v*), egg yolk emulsion (2% *w*/*v*), fibrinogen (0.28% *w*/*v*) and elastin (0.1% *w*/*v*) (Alcaide et al. 1999). DNase production was assayed on DNase agar (Oxoid, Hampshire, England). Gelatinase potency was evaluated using nutrient gelatin differential medium (Oxoid) that tests the ability of an organism to produce an exoenzyme that hydrolyzes gelatin. Haemolysin potency was evaluated using a modified plate assay technique described previously (Quindos et al. 1994). Briefly, 10 μL of suspensions (10^8 ufc mL^{-1}) were spotted onto human blood and fish blood agar made by mixing 70 mL of each blood with 1,000 mL (TSA) supplemented with 3% g NaCl (*w*/*v*). Fish blood was collected by caudal venous puncture from four or five healthy sea bass with weight over 100 g. The plates were incubated at 37°C for 24 h. Positive haemolytic potency was recorded by the presence of a distinct translucent halo around the inoculum area. The diameters of the zones of lysis and the colony were measured, and the ratio (equal to or larger than 1) was used as a haemolytic index to represent the intensity of haemolysin production by the tested strains. All the experiments were repeated three times. As phenotypic marker of the *trh* gene from *V. parahaemolyticus* isolates, the urease activity (Ure) was assayed through a conventional method, using Christensen's urea agar base (with 3% NaCl) which is used for rapid detection of urease activity (Hammack et al. 2001; Christensen 1946).

Infectivity tests

The 50% lethal dose (LD_{50}) test (with batches of 20 fish per dose) was conducted by intraperitoneal (i.p.) injection as previously described (Alcaide et al. 1999). Briefly, juveniles of *D. labrax* (weight 7 g and length 80 mm), kindly provided by the hatchery of higher Institute of Sea Monastir, were injected with 50 μL of a bacterial suspension containing 10^8 to 10^2 cfu mL^{-1}. The six strains were used in virulence test to quantify their virulence; the strains were grown overnight in TSA 3% NaCl at 30°C, from which one colony was subcultured in 40-mL fresh medium (TSB 3% NaCl) at the same condition for 16 h. The cells were harvested by centrifugation (5,000×*g*, 10 min), washed and

resuspended in PBS (0.01 M) to OD_{600} of 0.2 to 0.9, so that the bacterial concentrations were 10^2 to 10^8 cfu mL^{-1} determined by dilution plate method. Sterile PBS was injected i.p. into fish as control. The water temperature of the experiment was adjusted to 25°C. *D. labrax* juveniles were acclimated in sea water of 25°C for 4 to 5 days. Before infection, the juveniles of sea bream were fed with commercial food (ALLER FUTURA EX., ALLER AQUA, Aller, Christiansfeld, Denmark).

The juvenile fish were maintained at 25°C, 37‰ salinity and under continuous aeration. The juveniles were kept without feeding during the virulence test. Mortalities were recorded daily for 7 days and were only considered infected if *V. parahaemolyticus* was recovered from the assayed fish. The LD_{50} was calculated by simple method for estimating 50% endpoints (Reed and Muench 1938).

Survival in fish serum

The strains of *V. parahaemolyticus* were tested for their ability to resist the bactericidal activity of sea bass natural serum in a plate plaque assay. Sea bass serum was collected by centrifugation of total blood at 3,000 × *g* for 20 min at 4°C and stored at −20°C. The tests were conducted in 96-well microtitre dishes by mixing 100 μL of fresh serum or heat inactivated serum (44°C, 20 min) with 100 μL of bacteria suspension (10^6 cfu mL^{-1}) in PBS (pH 7). The assays were made by taking samples (10 μL) of the mixture (serum and bacteria) every 30 min for 4 h of incubation at room temperature (30°C). Viable counts were determined by drop plate on TSA 1% NaCl. Survival curve of all *V. parahaemolyticus* strains was determined using an Excel program.

Results

Biochemical and genetic identification

Table 1 describes the biochemical characterization of the strains isolated. The most outstanding character in the environmental strains is tryptophan deaminase and indole negative. According to API 20NE profiles (Table 2), four strains are identified as *V. parahaemolyticus*, and two are *Burkholderia gladioli*. The isolates which were initially identified as *V. parahaemolyticus* by conventional biochemical tests were further confirmed by PCR targeting species specific *toxR* (368 bp) gene (Figure 1). The two strains identified like *B. gladioli* were also positive for the *toxR* gene.

Characterization of *V. parahaemolyticus* isolates

In the present study, none of the strains showed beta haemolytic activity, but four strains were found to exhibit weak haemolysis, and all stains were negative for urease (Table 2). Most of the *Vibrio* isolates were producers of extracellular lipase, amylase, caseinase, DNase and gelatinase (Table 2). Experimentally infected fish showed external signs similar to those observed in outbreaks. During the determination of the LD50, all strains showing haemolytic activity were virulent and caused mortality during the infection tests. Pure culture of *V. parahaemolyticus* was isolated from liver and skin of moribund fish, and no moralities were detected in the control group. Signs of infection began to appear on the second day with strains S1 and S2, whereas for other strains, such signs were present only from the fourth day. The LD50 of the pathogenic strains ranged between 3.52×10^4 and 2.29×10^6 cfu g^{-1} fish (Table 2). Healthy fish infected with *V. parahaemolyticus* (S1 and S2) during virulence tests developed skin ulcers,

Table 2 Origin, hydrolytic enzymes and virulence factors of different strains isolated from internal organs diseased fish

Code	Biotype on API 20NE	Identification API 20NE (%)	Fish farm	Isolated from	Serum (D.L)	Hydrolytic enzymes								
						Lipase	DNase	Amylase	Gélatinase	Caseinase	Lecithinase	Haemolysis (D.L)	Haemolysis (H)	DL50
S1	7073345	99.9	I.F	Kidney	A	+	−	+	+	+	+	+	+	2.31×10^5
S2	5053345	99.9	O.F	Kidney	A	+	+	+	+	+	+	+	+	4.1×10^5
S3	7053304	99.9	O.F	Kidney	A	+	−	+	+	+	+	+	+	3.52×10^4
S4	7053345	99.9	O.F	Spleen	A	+	−	+	+	+	−	−	−	1.31×10^5
S5	7053734	Unacceptable	I.F	Spleen	B	+	−	+	+	−	+	−	−	2.29×10^6
S6	5053365	Unacceptable	I.F	Heart	B	+	+	+	+	+	+	+	+	Avirulent
ATCC 17802	5053345	99.9	−	Human	A	+	+	+	+	+	+	+	+	Avirulent
ATCC 43969	7053375	99.9	−	Human	B	+	+	+	+	+	+	+	+	ND

I.F, offshore farm; I.N, inshore farm; +, positive; −, negative; virulence, cfu $fish^{-1}$; ND, not determined; H, human erythrocytes; D.L, *Dicentrarchus labrax* erythrocytes; A, resisted to the serum; B, sensitive to the serum.

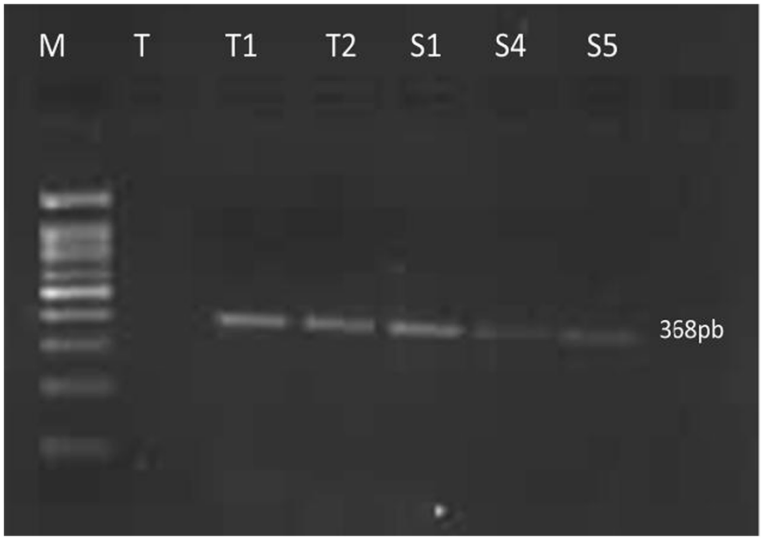

Figure 1 Reaction (PCR). M, molecular mass marker 100 bp; T, control; T1 and T2, controls + *V. parahaemolyticus* ATCC 43996 and ATCC 17802; S1, S4 and S5, environmental strains.

similar to those observed on the surface of sea bass collected from initial outbreaks in fish farms (Figure 2). With S4 and S5 strains, the fish do not show any signs of infection during the rest of experiments.

V. parahaemolyticus survival in fish serum

Only two strains isolated from sea bass (S5 and S4) were found to be serum sensitive. The survival curves of *V. parahaemolyticus* strains in sea bass serum are shown in Figure 3. The two strains showed different profiles: (A) strains that resisted the bactericidal action of the serum and were capable of proliferating, and (B) other strains that showed sensitivity to the serum and of which the percentage of survival decreased.

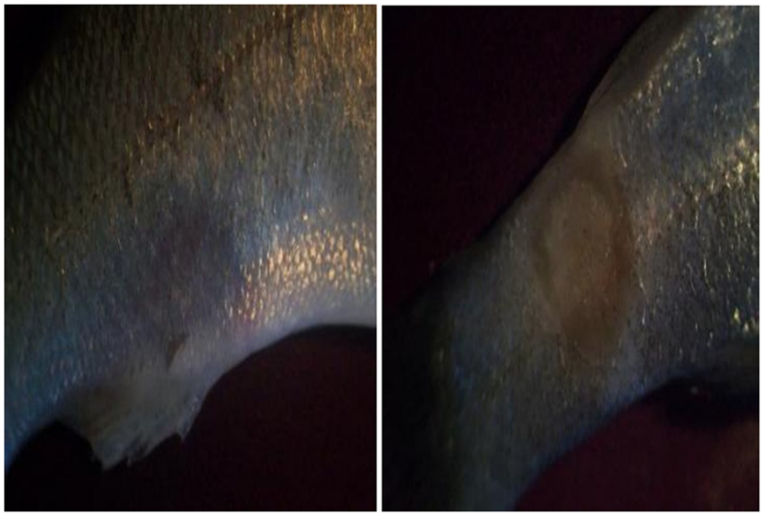

Figure 2 Signs of infection observed on *D. labrax* larvae including. Skin haemorrhages (left) and necroses (right) after infection tests.

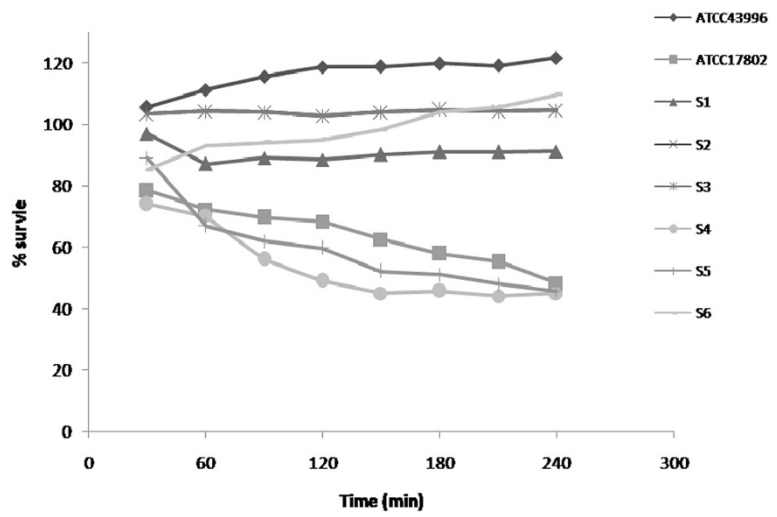

Figure 3 Survival curves. Six *V. parahaemolyticus* strains and the two reference strains (ATCC 43996 and ATCC 17802) in sea bass serum.

Discussion

During the spring of 2010, a wide mortality occurred in two aquaculture farms, starting in the offshore farm and then the same sign and the same mortality occurred in the inshore farm. The clinical signs of vibriosis affecting sea bass (*D. labrax*) included darkened body colour, white nodular skin lesion and sudden death with haemorrhages in the skeletal muscle. The six strains isolated during our study were reported to exhibit variation in one or two biochemical reactions, inducing errors in species identification (Dileep et al. 2003). The atypical reactions in environmental *Vibrio* spices may pose difficulties in organism biochemical identification (Karunasagar et al. 1996). *B. gladioli* is indole negative and lysine decorboxylation negative bacteria, knowing that most of the Tunisian *Vibrio* isolates were negative for indole production (Ben Kahla-Nakbi et al. 2007). Identifying *V. parahaemolyticus* strains through PCR-based method which targets the conserved region of *V. parahaemolyticus* such as *gyrB* and *toxR* gene is more efficient, reliable and faster compared to identifying through biochemical tests (Kim et al. 1999). Although *V. parahaemolyticus* is widely distributed in the coastal environments all over the world, most of the environmental strains are not pathogenic to humans, and only 1 to 2% of the environmental strains have shown to be positive Kanagawa TDH (Hervio-Heath et al. 2002; Robert-Pillot et al. 2004). In the present study, four of the isolates were found to exhibit weak haemolysis, and none of them was found to exhibit positive amplification for the *tdh* gene; none of the weak haemolytic strains were found to exhibit urease activity. It is likely that the *tdh* and *trh* positive strains are present in very low numbers along with strains negative for these genes (Dileep et al. 2003). The weak haemolysis points towards the presence of other virulence factors other than *tdh* in *V. parahaemolyticus*. This is confirmed during the determination of LD_{50}. All strains showing haemolytic activity are virulent and cause mortality during the infection tests even with the absence *tdh* and *trh* genes. *V. parahaemolyticus* is considered as a human pathogen more than a fish pathogen. Several cases of fish mortality due to this germ

are reported in Mediterranean coasts (Alcaide et al. 1999; Zorrilla et al. 2003). The organism has been associated with mortalities in Iberian toothcarp (*Aphanius iberus*) with the signs centred on external haemorrhages and tail rot (Austin and Austin 2007). Cultures regarded as intermediate between *V. alginolyticus* and *V. parahaemolyticus* were recovered from diseased milkfish (*Chanos chanos*) in the Philippines (Austin and Austin 2007). In *Penaeus monodon*, the organism has been implicated as a cause of red disease in India (Jayasree 2009). Pathogenicity has been established in *Solea senegalensis*, amberjack and eel by i.p. infections with LD50 dose of 6.3×10^5, 5×10^3 and 6.2×10^5 cfu $fish^{-1}$, respectively (Alcaide et al. 1999; Zorrilla et al. 2003). The corresponding value in abalone post-larvae was 3.5×10^5 cfu mL^{-1}, with the disease mirroring that of natural infections (Cai et al. 2007).

Many extracellular proteases are suggested to play important roles in the virulence of *Vibrio* spp. In a recent study (Lee et al. 2002), a protease was identified as the major virulence factor in a clinical *tdh*- and *trh*-negative *V. parahaemolyticus* isolate, and this enzyme showed cytotoxic activity on CHO and Vero cells (Ottaviani et al. 2005).

The enzymes' arsenal in these microorganisms gives them an edge in survival under adverse and stressful environmental conditions. Hydrolytic activities allow *Vibrio* strains the faculty to adhere to the epithelial cells of the juveniles of both *D. labrax* and *Sparus aurata*, to break the first barrier of natural defence and to colonize all internal organs, inducing external haemorrhages, necrotic eyes, deep ulcers, haemorrhagic liver, pale kidney and splenomegaly (Ben Kahla-Nakbi et al. 2009). In fact, virulence factors of non-toxigenic *V. parahaemolyticus* centre on proteases and adhesines. Serum plays an important role in the natural defence of fish against bacterial infections. The resistance to the serum is often associated with bacterial lipopolysaccharide (LPS). The high molecular weight portion of LPS is usually responsible for serum resistance in several bacterial pathogens (Amaro et al. 1995). Our results showed that virulent strains can survive the bactericidal action of the sea bass serum. Resistance to the bactericidal mechanisms of normal serum appears to be an important contributor to the virulence of fish-pathogenic *Vibrio* (Bordas et al. 1996).

However, the *V. parahaemolyticus* strains isolated during this outbreak were pathogenic to fish, but they did not possess any detectable virulence gene. The high diversity of strains belonging to the same species may explain the strain-specific expression of virulence factors and consequently the different data obtained from various geographic areas (Baffone et al. 2000).

Conclusions

This is the first report on the characterization and virulence of *V. parahaemolyticus* for sea bass in Tunisia and in Mediterranean coast. Vibriosis causes enormous economic loss in Tunisian aquaculture. The present study indicates that high abundance of extracellular hydrolytic enzyme profiles in the different strains isolated during this outbreak can be a main reason for the high mortality caused by this species. Knowing that, most of the fish larvae elevated in offshore farm are imported from several hatcheries in the Mediterranean Sea countries (France, Spain and Italy), and the inshore farm has its own hatchery. Moreover, genetic study is needed to view if strains responsible for such mortalities are imported with these larvae and introduced into Tunisian environment or they are already present in our environment (autochthon strains).

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
Faouzi Lamari carried out the infective tests,Amina Bakhrouf participated in the design of the study.

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